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# Benefits to host sea anemones from ammonia contributions of resident anemonefish

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ABSTRACT

Large ectosymbionts (especially fishes and crustaceans) may have major impacts on the physiology of host cnidarians (sea anemones and corals), but these effects have not been well quantified. Here we describe impacts on giant sea anemone hosts (*Entacmaea quadricolor*) and their endosymbiotic zooxanthellae (*Symbiodinium* spp.) from the excretion products of anemonefish guests (*Amphiprion bicinctus*) under laboratory conditions. Starved host anemones were maintained with anemonefish, ammonia supplements (= $NH_3$ gas and $NH_4^+$ ion), or neither for 2 mo. In the presence of external ammonia supplements or resident anemonefish, the zooxanthellae within host anemones increased in abundance (173% and 139% respectively), and provided the hosts with energy that minimized host body size loss. In contrast, anemones cultured with neither ammonia nor anemonefish harbored significantly lower abundances of zooxanthellae (84% of initial abundance) and decreased >60% in body size. Although they maintained higher zooxanthella abundances, anemones cultured with either ammonia supplements or resident anemonefish exhibited significantly lower ammonia uptake rates (0.065±0.005 μmol $g^{-1}$ $h^{-1}$, and 0.052±0.018 μmol $g^{-1}$ $h^{-1}$ respectively) than did control anemones (0.119±0.009 μmol $g^{-1}$ $h^{-1}$), indicating that their zooxanthellae were more nitrogen sufficient. We conclude that, in this multi-level mutualism, ammonia supplements provide essentially the same level of physiological contribution to host anemones and zooxanthellae as do live resident fish. This nutrient supplement reduces the dependence of the zooxanthellae on host feeding, and allows them to provide abundant photosynthetically-produced energy to the host.

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## 1. Introduction

Mutualisms are ubiquitous in nature, yet the mechanisms underlying most mutualistic interactions, especially in terms of the potential benefits to partner species, remain poorly understood and inadequately quantified (Bronstein, 1994; Bruno et al., 2003). In the association between anemonefishes (28 species of damselfishes in the genera *Amphiprion* and *Premnas*) and host sea anemones (10 species in the order Actiniaria, see Fautin and Allen, 1997), the obligate fishes cannot survive in nature without the safe haven they find among the anemones' tentacles (Mariscal, 1970; Allen, 1972; Fautin and Allen, 1997). The sea anemone hosts, on the other hand, are not obligate partners, and some species appear able to survive without anemonefishes (Godwin and Fautin, 1992; Fautin and Allen, 1997). Both partners can survive separately in laboratory aquaria (Fautin and Allen, 1997), but the anemones and also possibly the fishes grow faster when cultured together (Porat and Chadwick-Furman, 2005; N.E. Chadwick, unpublished data). In the wild, individuals of some host anemone species also survive and grow better when they harbor anemonefishes (Porat and Chadwick-Furman, 2004; Holbrook and Schmitt, 2005), but the underlying mechanisms through which anemonefishes benefit their hosts are not fully understood. Anemonefishes can defend some host anemones from cnidarian predators (Mariscal, 1970; Fricke, 1975; Fautin, 1991; Godwin and Fautin, 1992; Fautin and Allen, 1997; Porat and Chadwick-Furman, 2004). Physiological benefits also potentially accrue to host anemones through the utilization of resident anemonefish waste products (Fautin, 1991; Porat and Chadwick-Furman, 2005; Cleveland et al., 2008). We have demonstrated that anemonefish generate ammonia more rapidly than their host anemones can absorb it, thus providing a major source of external nitrogen for uptake by the endosymbiotic zooxanthellae within the hosts (Roopin et al., 2008). However, impacts of ammonia excretion by anemonefishes on the physiology of host anemones have not been well quantified.

All tropical sea anemones that host anemonefishes also harbor zooxanthellae (Dunn, 1981; Fautin, 1991), which supply the anemones with energy-rich photosynthetic compounds for respiration, growth, and reproduction (e.g., Steen, 1988; Achituv and Dubinsky, 1990; Whitehead and Douglas, 2003). In algal-cnidarian symbioses, zooxanthellae obtain inorganic nutrients from host catabolism (Szmant-Froelich and Pilson, 1984), host holozoic feeding (Steen, 1986), and the surrounding sea water (Muscatine, 1980; Wilkerson and Trench, 1986). The net excretory ammonia of host cnidarians often is insufficient to sustain zooxanthellae (Szmant-Froelich and Pilson, 1984; McAuley and Cook, 1994). For example, in the Red Sea coral *Stylophora pistillata*, excretory ammonia at steady state can potentially

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support only about 1/3 of the growth rate of its endosymbiotic zooxanthellae (Rahav et al., 1989). Cook and D'Elia (1987) first proposed that zooxanthellae may be nutrient-limited in hospite. Regulation of intracellular ammonium levels in the host potentially allows the host to control the biomass of resident zooxanthellae, and to maintain a steady-state ratio of symbiont to host in which neither outgrows the other (D'Elia and Cook, 1988; Falkowski et al., 1993). Several lines of evidence support this nitrogen-limitation theory, including the enhancement of dark carbon fixation in isolated zooanthellae following addition of ammonium chloride (Cook et al., 1994), and an increase in cnidarian zooxanthella densities with the addition of inorganic nitrogen (e.g., Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989; Hoegh-Guldberg, 1994). Thus, the contribution of ammonia waste products by anemonefishes (Roopin et al., 2008) can augment the capacity of sea anemone hosts to support and potentially increase their zooxanthella densities (e.g., Meyer and Schultz, 1985a,b; Porat and Chadwick-Furman, 2005), which in turn may provide additional photosynthetic energy to the host as needed when heterotrophic food sources are limited (Roberts et al., 1999).

The direct transfer of nitrogen from anemonefish to sea anemone host tissues has been demonstrated by Cleveland et al. (2008) using stable isotope markers. However, the physiological impacts of this nutritional benefit, and the extent to which nitrogen versus other fish-related factors contribute to host body size, remain unknown. Here we compare the impacts of symbiotic anemonefish presence versus ammonia supplements on the body size and zooxanthella population dynamics of starved host sea anemones under laboratory conditions.

## 2. Methods

### 2.1. Study organisms and maintenance

Giant sea anemones *Entacmaea quadricolor* (Rüppell and Leuckart, 1828) were transported to Auburn University in 2006 from Waikiki Aquarium (Hawaii, USA), where they had been propagated via clonal replication from individuals collected in Palau in 1985. All of the zooxanthellae contained within the endodermal tissues of these host sea anemones belonged to *Symbiodinium* clade C1 (Roopin, 2007). Anemonefish *Amphiprion bicinctus* Rüppell, 1828 also were transported to Auburn in 2006 from Oceans Reefs and Aquariums (ORA), an aquaculture facility of Harbor Branch Oceanographic Institution at Fort Pierce, Florida, USA, where they had been propagated from brood stock collected in Saudi Arabia in 2000. One anemone was placed on each side of a rigid plastic screen (2 cm mesh) in each of 12 identical closed-system aquaria ($n$=24 anemones total), together with 1 - 2 anemonefish per anemone ($n$=30 anemonefish total). All fish and anemones were acclimated to these culture conditions for at least 4 mo prior to experiments, and grew actively during this period. Each closed-system aquarium circulated 160 L of artificial seawater (Reef Crystals, Aquarium System, Inc., Ohio, USA) between an upper tank (77 cm×32 cm×33 cm) containing the animals, and a lower sump (77 cm×32 cm×33 cm) with filters. Water flowed into the upper tank from the sump alternately through 2 pipe outlets using a SCWD–wave maker (3iQ Ventures LLC, Manhattan Beach, California, USA). Each sump contained a protein skimmer, live rock, and macroalgal cultures as filters, and both upper tanks and sumps contained a layer of fine sand. Anemones were allowed to attach to pieces of flat coral rock that were placed in each upper tank. All systems were maintained at 34 - 35 ppt salinity, temperature of 26 °C, and a 12 h light : 12 h dark photoperiod. Concentrations of dissolved ammonia in the tanks were consistently low, <0.5 μmol $L^{-1}$. Over each aquarium was suspended a 6-bulb TEK-LIGHT™T5 high output fluorescent light, with a combination of 3 39W T5 Midday 6000K, and 3 39W T5 Pure actinic Giesemann PowerChrome fluorescent bulbs, which provided photosynthetically-active radiation (PAR) of about 200 μmol quanta $m^{-2}$ $s^{-1}$ at the bottom of the aquarium to 800 μmol quanta $m^{-2}$ $s^{-1}$ at the water surface (QSL-2101 Scalar PAR Sensor, Biospherical Instruments, San Diego, California, USA), equivalent to PAR at 7-20 m depth on coral reefs in the Red Sea (Stambler and Dubinsky, 2005) where these organisms occur (Fautin and Allen, 1997; Chadwick and Arvedlund, 2005).

### 2.2. Experimental design

All anemones were fed weekly to satiation with small pieces of fish or shrimp. Anemonefish were fed each morning to satiation with a combination of dry pellets (Formula one, AquaPet Americans, Utah, USA) and frozen foods (copepods, brine shrimps, mysids).

To assess effects of ammonia enrichment on starved sea anemones, each individual anemone was assigned haphazardly to 1 of 3 treatments for a period of 2 mo: (1) without anemonefish in nutrient-poor seawater, referred to as the control treatment ($n$=8 anemones), (2) with 1 - 2 anemonefish in nutrient-poor seawater, referred to as the anemonefish treatment ($n$=8 anemones), and (3) without anemonefish in ammonia-enriched seawater (daily ammonia treatment of ~10 μmol $L^{-1}$ ammonia for 1 - 1.5 h), referred to as the ammonia treatment ($n$=8 anemones). Identical treatments were assigned to both sides of each aquarium system, to avoid treatment effects from one side of the mesh barrier to the other. Each aquarium system was assigned randomly to a treatment using a random number generator. During the experiment, food was withheld from the anemones, but the anemonefish were fed daily with dry pellets only (Formula one, AquaPet Americans, Utah, USA). Pellets were introduced to the fish one by one and their feeding behavior was monitored carefully. Uningested pellets were removed from the tanks, and the anemones did not ingest any of the food intended for the fish. After 2 mo, nutrient supply treatments were reversed as follows: (1) anemonefishes were removed from the anemonefish treatment, (2) daily ammonia supplements were stopped for the ammonia treatment, and (3) 1 - 2 anemonefish per anemone were added to the control treatment. These reversed treatments were maintained for an additional 3 wk, then all animals were returned to their original culture conditions.

### 2.3. Size changes in unfed sea anemones

To assess patterns of change in body size during starvation, each sea anemone was photographed at the start of the experiment and after 2 mo, prior to treatment reversal. Photographs were taken only when anemones were fully expanded. Three individuals, 1 in each treatment, were excluded from the size analysis because they consistently remained contracted, and could not be measured accurately. Photographs were scanned into a computer, and the software program Image Tool (Ver.3.00, UTHSCSA) was used to determine the long and short axial lengths of the sea anemones (tentacle tip to tentacle tip, an approximation of tentacle crown diameter). The area covered by the tentacles of each anemone (tentacle crown surface area) was regarded as an oval and was estimated as (long axial length) × (short axial length) × π/4 (after Hirose, 1985). Changes in sea anemone body sizes were estimated by calculating the final tentacle crown surface area of each as a percentage of initial area.

### 2.4. Zooxanthella parameters

To assess the condition of the zooxanthellae within host sea anemones in each treatment during starvation, we determined the abundance, cell division rate (mitotic index), and chlorophyll *a* content of the zooxanthellae every 2 - 3 wk during the study. During each sample period, 3 tentacle tips (2 - 3 cm long each) were removed from each anemone and immediately analyzed. Sampling did not appear to adversely affect the anemones, as each possessed many tentacles and rapidly regenerated lost tentacle tips (Porat and Chadwick-Furman, 2005). Each tentacle tip was blotted dry and

weighed to obtain its wet mass, then homogenized in 2 mL of artificial seawater with a 5 mL Wheaton tissue grinder, and the homogenate centrifuged at high speed (1000×*g*) on a 5415D Eppendorf centrifuge (Eppendorf North America, Westbury, New York, USA) for 5 min to pellet the algal cells. The supernatant was removed by Pasteur pipet, and the algal pellet was re-suspended in 2 mL of seawater. This procedure was repeated at least 3 times to produce a pellet consisting of mostly zooxanthellae and almost no animal tissue (after Cook et al., 1988). The zooxanthella pellet then was suspended in 2 mL of seawater, and a 1 mL sub-sample of this zooxanthella cell suspension was transferred to a separate vial for chlorophyll *a* analysis (see below). The remaining cell suspension was diluted with seawater to produce densities between $3.0\times10^5$ and $7.4\times10^6$ cells $mL^{-1}$ and aliquots were removed for cell counts. Zooxanthella numbers were determined from 4 replicate counts using a Hausser Scientific hemacytometer (after D'Elia and Cook, 1988), except for during week 6 when technical problems led to only 3 counts per sample.

The proportion of cells undergoing division in each sample was used to calculate a zooxanthella mitotic index (McDuff and Chisholm, 1982). Algae were counted under a phase contrast microscope (400×), and cells were scored as dividing if they were doublets up to the stage of separation of daughter cells (after Wilkerson et al., 1983; Cook et al., 1988). The specific growth rate (μ) of the zooxanthellae was calculated based on the maximum MI value observed, using the equations of Wilkerson et al. (1983). In a preliminary experiment, the mitotic activity of zooxanthellae in fed individuals of *Entacmaea quadricolor* was measured repeatedly at 4 h intervals over a 24 h period, revealing a peak in zooxanthella division rate at about 07:30 h, 1 h after the start of the light period at 06:30 h each day. Therefore, tentacle sampling routinely was conducted at 07:30 - 08:00 h throughout the experiment.

Chlorophyll *a* levels were assessed by extracting a 1 mL aliquot of zooxanthella cell suspension (described above) with 90% acetone overnight at 4 °C, centrifuging the acetone extract, and reading absorbance on a spectrophotometer. Chlorophyll *a* concentrations were calculated using the equations of Jeffrey and Humphrey (1975).

### 2.5. Rates of ammonia uptake by sea anemones

To determine effects of the treatments on rates of ammonia uptake by the anemones, measurements were made after 2 mo of starvation, prior to treatment reversals. Ammonia flux in 4 haphazardly-selected sea anemones from each of the above 3 treatment groups was determined in the light under laboratory conditions (see Roopin et al., 2008 for details). Rates of ammonia uptake by the anemones were computed from the regression lines of change in ammonia concentration over time for each run, using all 4 samples. Changes in water volume due to sampling were incorporated into all calculations. At the end of each experimental run, anemones were removed from the experimental vessels and returned to their original aquaria. This procedure did not appear to greatly affect the anemones: some contracted their tissues when transferred to the experimental vessels, but all re-expanded and attached their basal disks to the glass within the first 5 - 10 min of the experiment. Since the anemones were maintained under constant irradiance throughout the light period (12 h) each day, it was assumed that any "time of day" effect would be minor, and thus all experiments were conducted during mid-day (after Roopin et al., 2008). Photographs of the anemones taken before measurement of ammonia uptake were scanned into a computer, and used to determine anemone tentacle crown diameters, as described above. Anemone wet mass was estimated from the relationship between wet mass and tentacle crown diameter for individuals of *Entacmaea quadricolor* from a separate study: $y=0.20\ x^{2.10}$, where y=wet mass in grams and x = tentacle crown diameter in cm (N=9 anemones, $r^2=0.87$, $p<0.05$; Godinot and Chadwick unpublished data). The wet mass of anemones in the present study was estimated to be 21.9±2.6 g (range=12.6 - 44.8 g, N=12 anemones).

### 2.6. Statistical analyses

All statistical analyses were conducted using SPSS 15.0. Normality and homogeneity of variance were examined using the Shapiro–Wilk statistical test for $n<50$. Differences in ammonia uptake rates among the treatment groups were tested using one-way analysis of variance (ANOVA), followed by post-hoc pairwise comparisons (LCD and Sidak). Differences among treatment groups in zooxanthella abundance, chlorophyll *a* content, and cell division rates were tested using repeated measures ANOVA with time as the repeated measure, followed by pairwise comparisons of estimated marginal means (Bonferroni adjusted). The Bonferroni correction was applied to all significance levels. All results were considered significant at $p<0.05$, and are presented as means±1 standard error unless otherwise indicated.

## 3. Results

### 3.1. Size changes in unfed sea anemones

During the 2 mo starvation experiment, the anemones in all 3 groups decreased significantly in body size (repeated measures ANOVA, $F_{(1,18)}=42.52$, $p<0.005$, Fig. 1A). A significant interaction effect between time and treatment group ($F_{(2,18)}=4.911$, $p<0.02$) indicated that anemone sizes varied among treatment groups in their response to starvation.

Analysis of variation among groups in the amount of body size lost by the anemones indicated that size loss varied significantly with treatment (ANOVA, $F_{(2,18)}=7.36$, $p<0.005$). When compared to their initial sizes, anemones in the control treatment decreased in size

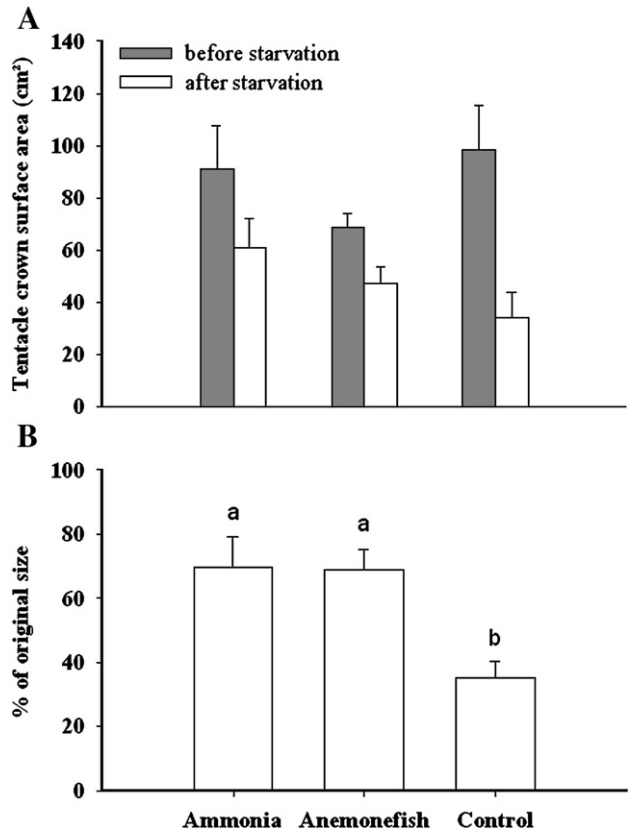


**Fig. 1.** Variation in the body size (expressed as tentacle crown surface area) of unfed giant sea anemones *Entacmaea quadricolor* among laboratory treatments: (A) Initial (black bars) and final (white bars) body size after 2 mo of treatments. Data are shown as means±1 standard error. (B) Final size expressed as a percent of initial size. $n=7$ anemones per treatment. Treatments with different letter values denote significant differences at $p<0.05$ (ANOVA followed by post-hoc pairwise comparisons).

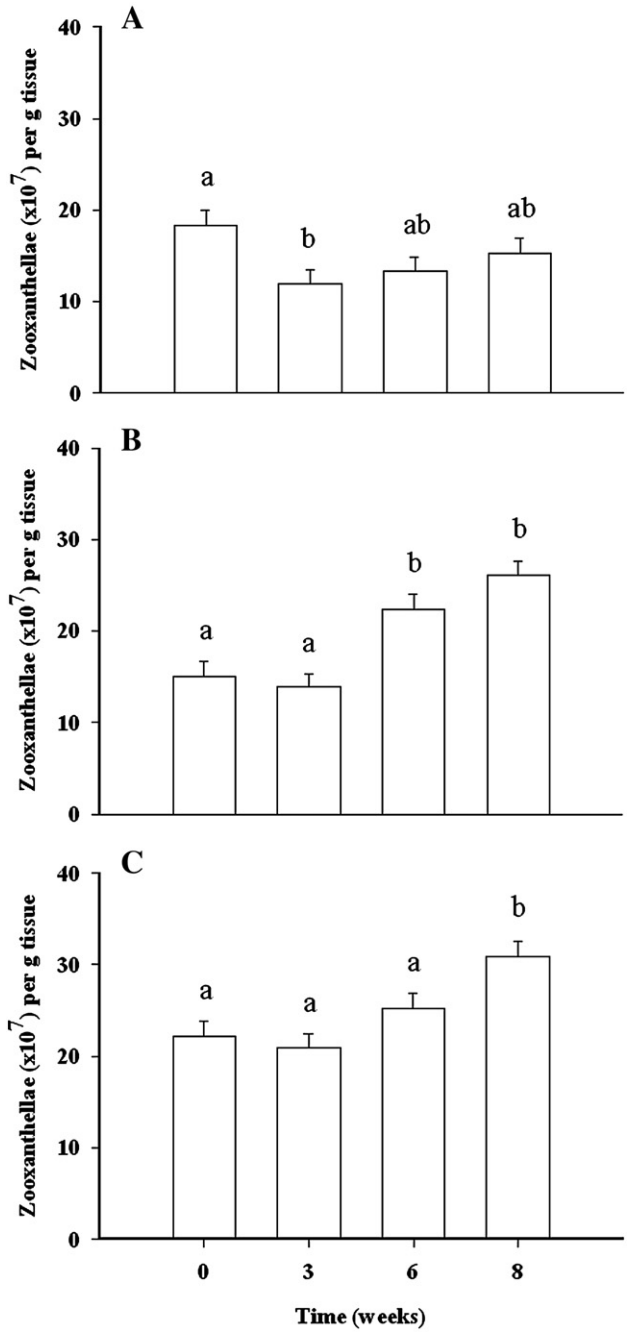


**Fig. 2.** Variation in the abundance of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* among laboratory treatments: (A) control, (B) ammonia-enriched, and (C) anemonefish. $n=8$ anemones per treatment. Data are shown as means±1 standard error. Times with different letter values denote significant differences at $p<0.05$ (repeated measures ANOVA followed by Bonferroni-adjusted pairwise comparisons of marginal means).

twice as much (64.75±4.9% of original size) as did anemones in the other two treatments (with anemonefish, 31.04±6.33% of original size, and with ammonia supplement, 30.35±9.61% of original size). This loss was significantly greater than that in the latter 2 treatments (LSD and Sidak post hoc analyses, $p<0.004$), which did not differ significantly in percent size loss from each other ($p=0.947$, Fig. 1B).

### 3.2. Zooxanthella parameters

Zooxanthella abundance within the sea anemone tentacles varied significantly over time in all 3 treatments during the 8 wk starvation period (repeated measures ANOVA, $F_{(3,63)}=19.24$, $p<0.005$). There was a significant interaction effect between time starved and treatment group ($F_{(6,63)}=6.00$, $p<0.005$), indicating that the rate of change in zooxanthella abundance differed significantly among the treatments ($F_{(2,21)}=23.29$, $p<0.005$). The abundance of zooxanthellae in the control anemones decreased significantly from original levels during the first 3 wk of the experiment (from $18.3\pm1.6\times10^7$ to $12.0\pm1.4\times10^7$ cells per g tentacle tissue, pairwise comparisons test, $p<0.005$, Fig. 2). This initial reduction in algal abundance was evident in the increasingly pale appearance of these anemones. In contrast, zooxanthella abundances in the ammonia treatment did not change significantly during the first 3 wk of starvation ($p=1.00$), and then increased significantly from $13.9\pm1.4\times10^7$ cells per g tentacle tissue at wk 3 to $22.4\pm1.6\times10^7$ at wk 6 of starvation ($p<0.001$, Fig. 2). Anemones in the anemonefish treatment also did not significantly alter their zooxanthella abundances during the first 3 wk of starvation ($p=1.00$), and then significantly increased them from $25.3\pm1.7\times10^7$ cells per g tentacle tissue at wk 6 to $30.9\pm1.6\times10^7$ at wk 8 ($p<0.028$, Fig. 2). After reversal of the treatments at the end of wk 8, the anemones in all groups significantly altered their zooxanthella abundances in the opposite direction (repeated measures ANOVA, $F_{(4,84)}=13.05$, $p<0.005$). Addition of anemonefish to the control anemones caused a significant increase in their zooxanthella abundances (pairwise comparisons test, $p<0.001$) to a much higher level than at the beginning of the experiment (Fig. 3). In the other 2 groups, the cessation of ammonia supplements and removal of resident anemonefish resulted in a significant decrease in zooxanthella abundance ($p<0.015$ and $p<0.001$ respectively) to below their levels at the beginning of the experiment.

Prior to the start of the starvation experiment, the rate of cell division by zooxanthellae in fed anemones followed a strong diel cycle, with a division peak early in the morning, similar to patterns described for other sea anemones under both field and laboratory conditions (Cook et al., 1988; Smith and Muscatine, 1999). The mitotic activity of zooxanthellae began to increase approximately 1 h before dawn (05:30 h), peaked at approximately 1 h after dawn (07:30 h), and then by 12:00 h had declined to a slow rate that remained fairly constant until the next morning (Fig. 4A). The mean mitotic index (MI) at each sample time ranged from 1.5 to 4.9% during the day ($n=3$ anemones tested, range of SD=0.51-0.93, Fig. 4A). The specific growth rate (μ) of the zooxanthellae was calculated as 0.0478 $d^{-1}$. No samples were observed with zero dividing cells during the off-peak hours, but since most mitotic activity occurred during a distinct peak each day, the zooxanthellae were interpreted as displaying phased division.

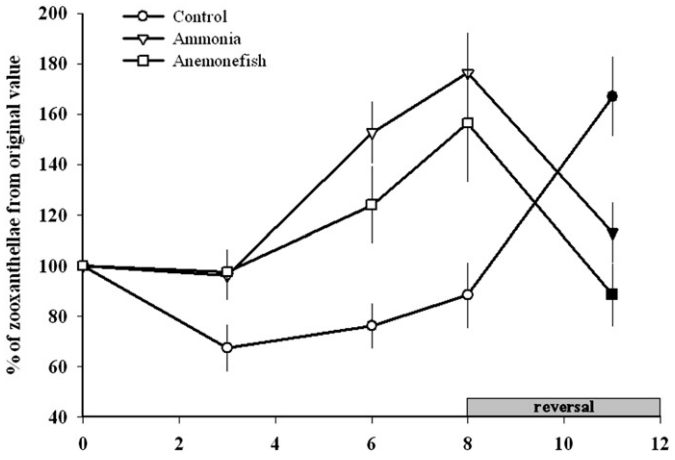


**Fig. 3.** Variation in the abundance of zooxanthellae in unfed giant sea anemones *Entacmaea quadricolor* among laboratory treatments, expressed as percent of initial abundance. Open symbols represent the period during treatments (weeks 0-8), and closed symbols the period after treatments were reversed (weeks 8-11). $n=8$ anemones per treatment. Data are presented as means±1 standard error.

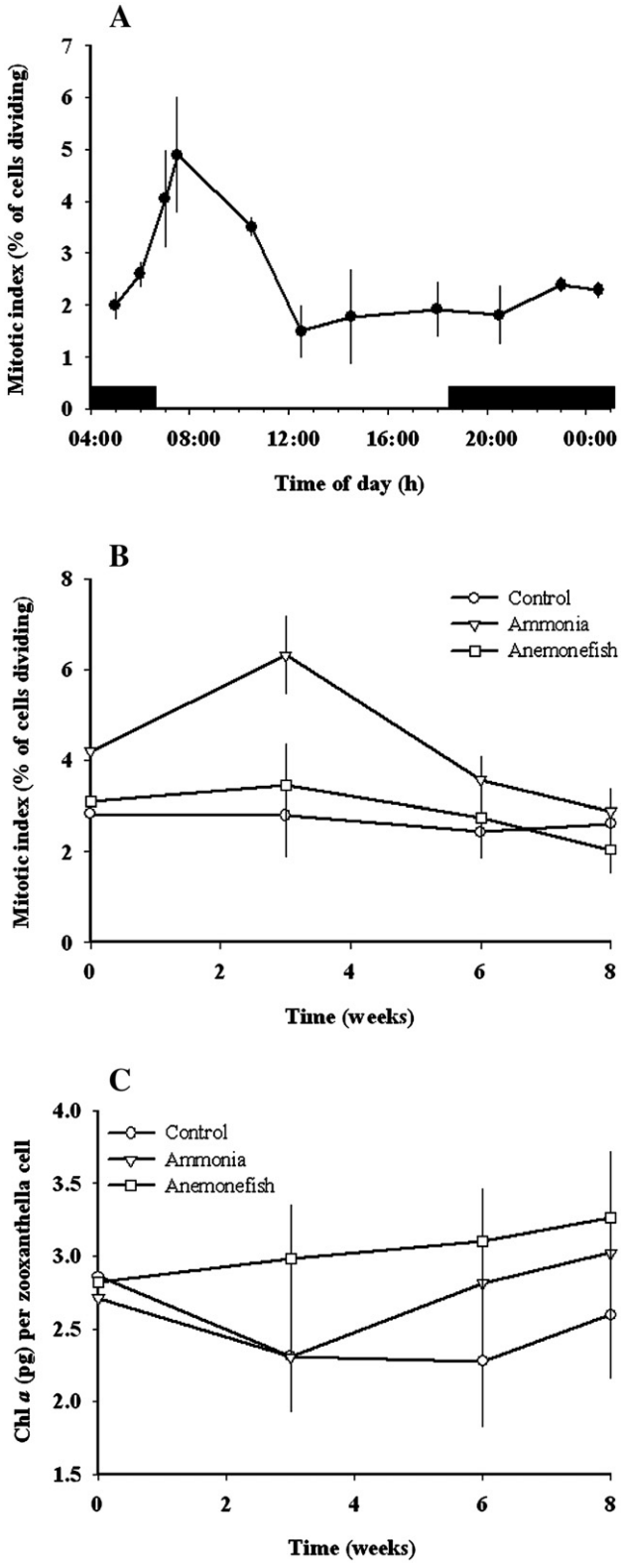


**Fig. 4.** Zooxanthella cell division and pigment content in giant sea anemones *Entacmaea quadricolor* under laboratory conditions: (A) Diel cycle of mitotic index (% of zooxanthella cells dividing) when fed weekly and subjected to a 12 h light : 12 h dark cycle, in which light began each day at 06:00 h and ceased at 18:00 h. $n=3$ anemones examined during each time interval. (B) Long-term change in mitotic index during starvation in 3 treatments over 8 weeks. (C) Long-term change in chlorophyll *a* content per zooxanthella cell during starvation in 3 treatments over 8 weeks. Each data point represents the mean value of the 8 anemones in each treatment group at each time point. Data are presented as means ± 1 standard error.

After the start of the starvation experiment, the MI of zooxanthellae in the control anemones remained constant, but in both treatments with a supply of inorganic nitrogen (artificial or through fish excretion), a temporary increase in mitotic activity occurred after 3 wk (Fig. 4B). By wk 6, the mitotic indices had declined, and by wk 8, the anemonefish and ammonia treatments were below their original levels. A repeated measures ANOVA indicated that the MI varied significantly over time ($F_{(3,63)}=5.07$, $p<0.005$), while differences among treatment groups were not significant ($F_{(2,21)}=2.615$, $p=0.097$).

Values of chlorophyll *a* per zooxanthella cell did not change significantly during the 2 mo experimental period (repeated measures ANOVA, $F_{(3,63)}=1.180$, $p=0.325$). During the first 3 wk, chlorophyll *a* levels in the control treatment ranged from 2.86 ± 0.29 to 2.31 ± 0.38 pg chl *a* cell$^{-1}$, in the ammonia treatment from 2.71 ± 0.29 to 2.30 ± 0.38, and in the anemonefish treatment from 2.82 ± 0.29 to 2.98 ± 0.38 (Fig. 4C). Final chlorophyll levels at wk 8 in the control anemones were 2.59 ± 0.47 pg chl *a* cell$^{-1}$, in the ammonia treatment they were 3.02 ± 0.47, and in the anemonefish treatment they were 3.26 ± 0.47 (Fig. 4C).

### 3.3. Rates of ammonia uptake by sea anemones

In control vessels without animals, ammonia concentrations did not change significantly throughout the experiments (t-test, t = 0.37, df = 5, $p=0.71$). Thus, there was no significant ammonia loss due to

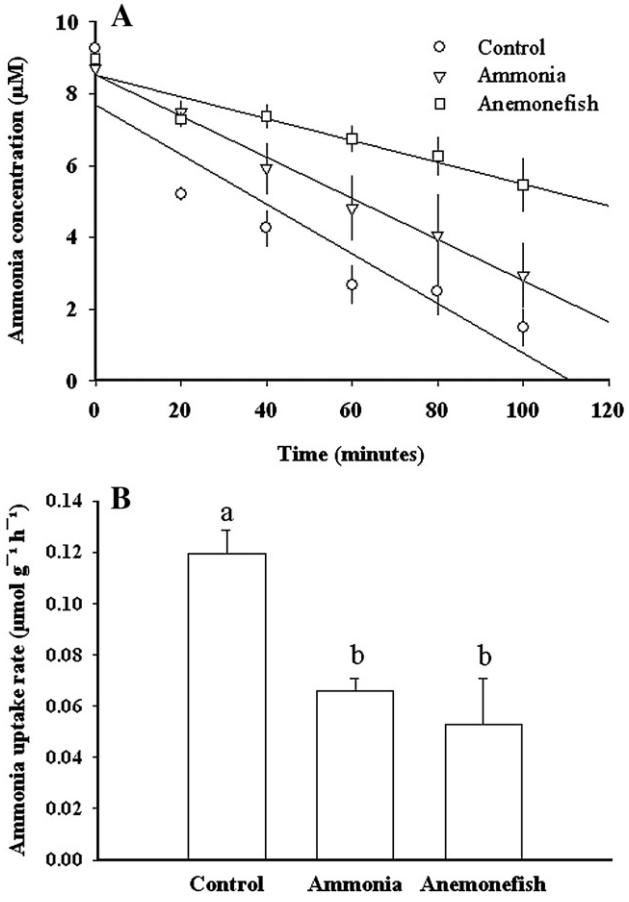


**Fig. 5.** Ammonia uptake rates of unfed giant sea anemones *Entacmaea quadricolor* after 2 mo in 3 laboratory treatments. (A) Time course of ammonia clearance among treatments. Lines were fitted by regression analysis, using all 4 values at each time point. (○) individuals that received a daily ammonium treatment ($y=-0.0574x+8.5193$, $r^2=0.99$), (□) individuals kept with resident anemonefish ($y=-0.0304x+8.5323$, $r^2=0.91$), (Δ) control anemones that received no supplements and no fish ($y=-0.0694x+7.7011$, $r^2=0.8594$). (B) Variation in rates among treatments. $n=4$ anemones tested for ammonia uptake from each treatment. Data are presented as means ± 1 standard error.

uptake by microbes or other reasons, and adjustments were not made to the data. Similar conclusions were reached by Bray et al. (1986) and Roopin et al. (2008).

When exposed to light, all of the anemones removed ammonia from solution (Fig. 5A). Rates of ammonia uptake by the sea anemones in ammonia-enriched seawater (10 μmol $L^{-1}$) varied significantly with treatment group (ANOVA, $F_{(2,11)}=8.277$, $p<0.009$). Post hoc analyses indicated that the ammonia uptake rate of anemones in the control treatment (0.119±0.009 μmol $g^{-1}$ $h^{-1}$) was about twice as rapid and significantly higher than that of anemones from the anemonefish or ammonia treatments, which did not differ significantly from each other (0.052±0.018 μmol $g^{-1}$ $h^{-1}$, and 0.065±0.005 μmol $g^{-1}$ $h^{-1}$, $p<0.05$, Fig. 5B).

## 4. Discussion

We demonstrate here that artificial ammonia supplements reduce size loss and enhance zooxanthella content in starving host sea anemones as much as does the presence of resident anemonefish. In addition, anemones cultured with either ammonia supplements or fish both experience less nitrogen deficit, evidenced in their lower ammonia uptake rates compared to control anemones deprived of either. Thus, the ammonia contributed by resident anemonefish appears to provide a major physiological benefit to sea anemone hosts and zooxanthellae in this three-way mutualism. This nutrient contribution by resident fish allows host anemones to maintain a stable metabolism when heterotrophic nutritional input is low, thus potentially increasing the range of environmental disturbances that host anemones can withstand.

The decreased abundance of algal symbionts during starvation of the control anemones observed here was similar to the pattern known for some other zooxanthellate cnidarians (e.g., Clayton and Lasker, 1984; Cook et al., 1988; Titlyanov et al., 2000). Zooxanthella responses to host starvation differ among associations, and also may involve increases in algal abundance (e.g., Muller-Parker, 1985; Hoegh-Guldberg and Smith, 1989) or no change (Kevin and Hudson, 1979; Fitt et al., 1982). Here only the anemones without ammonia supplements reduced their zooxanthellae during 3 wk of initial starvation, thus those in the fish and ammonia-supplement treatments appeared to receive sufficient nutrients to maintain their algal symbiont populations. As the starvation experiment progressed, higher zooxanthella abundances in anemones with fish or ammonia input appeared to contribute substantially to the maintenance of host body size. The similar patterns of change in algal abundance that were observed for anemones in both the anemonefish and ammonia treatments indicate that ammonia (or lack of it) is a primary factor in limiting zooxanthella abundance and thus host ability to tolerate low heterotrophic input. In this way, the anemonefish appear to provide physiological benefits to their host anemones in large part through their contribution of excreted ammonia.

Addition of anemonefish to the control anemones resulted in recovery of their zooxanthella populations during treatment reversal, while the opposite pattern occurred in the other groups, thus confirming that ammonia availability through fish presence is a primary factor controlling the zooxanthellae in these host anemones. When food is scarce, cnidarian host feeding and catabolism often are insufficient to supply all of the nitrogen requirements of growing zooxanthellae (Rahav et al., 1989). Thus, when input from heterotrophic feeding is low and the host's ability to support its zooxanthellae population is compromised, lack of an alternative nitrogen source results in a rapid depletion of the host's essential body reserves (e.g., Cook et al., 1988; Beaver, 1996; Roberts et al., 1999). The external ammonia contributions of resident anemonefish appear to provide such a source, and to reduce the dependence of zooxanthellae on host feeding, thereby allowing starved anemone hosts to maintain high zooxanthella densities (e.g, Summons et al., 1986; Hoegh-Guldberg and Smith, 1989). This response is similar to that known for some stony corals, in which the zooxanthellae become nitrogen-limited at high abundance, but when external ammonia supplements are added, they allow maintenance of even higher zooxanthella abundance (Hoegh-Guldberg and Smith, 1989).

The temporary stimulation of zooxanthella division rates that we observed in some of our treatments also occurs in other cnidarians exposed to external ammonia supplements (Cook et al., 1988; Muscatine et al., 1989; Hoegh-Guldberg, 1994; Smith and Muscatine, 1999). However, both external ammonia level and the nutritional status of the host appeared to have little overall effect on the algal division rates observed here, since the mitotic index remained low in all treatments throughout the remainder of our experiment. The mechanisms that maintain symbiont densities in cnidarian hosts are poorly understood, and may involve regulation by the host so that neither symbiotic partner outgrows the other (Cook, 1985; Smith and Muscatine, 1999). Muscatine and Pool (1979) proposed 3 pathways through which hosts regulate their zooxanthella numbers: (1) expulsion of algal cells, (2) digestion of excess algae, and/or (3) pre-mitotic inhibition of algal growth via restricted access to essential nutrients, or through production of growth-inhibiting factors (Muscatine, 1967). Since the division rates of zooxanthellae were consistently low among our treatments, the gradual increases we observed in zooxanthella abundance per g host tissue more likely were facilitated by decreases in either host biomass, or expulsion rates of zooxanthellae, or both (Stimson and Kinzie, 1991; Baghdasarian and Muscatine, 2000; Muller-Parker and Davy, 2001). Although, the intertidal sea anemone *Anthopleura elegantissima* has been suggested to control its symbiont population via expulsion (McCloskey et al., 1996), in most associations this path does not appear to be a major zooxanthella regulation factor, and at most it can act as a "fine tuning" mechanism for regulation at steady-state (e.g., Hoegh-Guldberg et al., 1987; Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989; Baghdasarian and Muscatine, 2000). As such, a hypothetical reduction of the typically low algal expulsion rate (Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989) is not likely to facilitate any substantial increase in net zooxanthella numbers under starvation stress. Some cnidarians such as stony corals (Titlyanov et al., 1996) and soft corals (Benayahu and Schleyer, 1998) may partly regulate symbiont density by digesting their algae, but no evidence exists for this regulatory mechanism in sea anemones. Thus, we conclude that during early starvation, a temporary ammonia-stimulated increase in the rate of algal cell division occurred in the anemonefish and ammonia treatments here, which delayed any decrease in algal numbers due to lack of host food. Later, when algal growth rates returned to normal, the observed increases in zooxanthella density likely were due to a combination of the loss of anemone host tissue (i.e. a decrease in algal habitat size) and maintenance of high zooxanthella densities by enhanced nutritional status, possibly via reduced rates of algal cell mortality, digestion, and/or expulsion. This conclusion is in agreement with that of Smith and Muscatine (1999) that no single mechanism regulates the standing stock of zooxanthellae, but that a combination of processes interact to maintain zooxanthella levels compatible with the metabolic state of the host and nutrient availability in the external environment.

Our observation of no significant variation in per-zooxanthella chlorophyll content with the nutritional state of *E. quadricolor* sea anemones is similar to that of Clayton and Lasker (1984), who found that zooxanthella chlorophyll content did not differ between fed and starved sea anemones of the species *Aiptasia pallida*. In contrast, Cook et al. (1988) revealed that low nutrient availability to zooxanthellae within the same sea anemone host may influence their ability to synthesize chlorophyll. Nutrient limitation in our anemones thus appeared to impact zooxanthella population dynamics and ammonia uptake rates rather than chlorphyll amounts within individual zooxanthella cells. The above responses indicate that the mechanisms controlling how changes in nutrient conditions affect sea anemones and their zooxanthellae appear to vary widely.

The magnitude of response by giant sea anemone hosts to anemonefish nutrient supplementation under natural conditions on the coral reef may differ from that observed here in the laboratory, and also likely varies among coral reef habitats and species combinations. However, our laboratory conditions simulated those in the field, in that seawater in the tanks contained very low levels of inorganic nitrogen (~0.5 μmol $L^{-1}$ ammonia), and high levels of water flow were created over the anemones using powerful pumps (see Methods). Nutrient dynamics also may be influenced by variation in physiological performance among different types of *Symbiodinium* present in the host (e.g., Chang et al., 1983; Hoegh-Guldberg and Smith, 1989; Goulet et al., 2005). Here, all examined host individuals were cultured under the same conditions and harbored only *Symbiodinium* C1 symbionts (Roopin, 2007), therefore it is unlikely that the impacts of nutrient transfer varied due to differences in the physiological performance of the algal symbionts.

The slower loss of animal biomass in treatments with supplemental nutrients compared to the starved controls indicates that the latter anemones experienced a more severe energetic deficit and therefore rapidly depleted their body reserves. Similar conclusions have been drawn for the sea anemone *Anemonia viridis* (Beaver, 1996; Roberts et al., 1999): starved individuals that received constant nutrient supplementation (20 μmol $L^{-1}$ ammonia) exhibited a positive daily growth, in contrast to the negative growth observed in anemones with no nutrient supplements. Differences in the concentration of the ammonia supplement provided, as well as variation in physiology between the anemone species may account for the positive growth reported by Roberts et al. (1999) versus the diminished size loss that we observed here. Despite these differences, nutrient supplementation retarded the loss of host body size in both studies. Given that the ammonia supplied by anemonefish substantially delays the effects of starvation in our host anemones under laboratory conditions, it may also in large part cause the enhanced growth rates of fish-hosting sea anemones under natural conditions on coral reefs (Porat and Chadwick-Furman, 2004, 2005; Holbrook and Schmitt, 2005). In the field, host anemones may receive less ammonia from their resident fishes than under laboratory conditions, since the fish range up to 1 m or more away from the host while foraging on zooplankton (reviewed in Roopin et al., 2008). On the other hand, while anemones in this study contained only 1-2 relatively small anemonefish of about 11 g wet mass each, in the field giant sea anemones can be occupied by 2 adult anemonefish that weigh up to 64 g each (Chadwick and Arvedlund, 2005; reviewed in Roopin et al., 2008).

Starved individuals of *E. quadricolor* and their zooxanthellae develop a nitrogen deficit, as indicated by the significantly faster ammonia uptake by our starved control anemones in comparison with the nutrient-supplemented anemones examined here, and by fed individuals in a separate study (Roopin et al., 2008). Zooxanthellae in other unfed cnidarian hosts also are known to absorb and retain external inorganic nutrients at a significantly faster rate than do those in fed hosts (e.g., Szmant-Froelich and Pilson, 1984; D'Elia and Cook, 1988). Resident anemonefish appear to contribute slightly but not significantly more to host nutrition than does artificially added ammonia, as indicated by the low ammonia uptake rates of anemones cultured with fish. Thus, the presence of anemonefish may support an accumulation of nutrient stores by host zooxanthellae, in addition to supporting the production of new algal cells (Muscatine et al., 1989; Muller-Parker et al., 1994).

An additional nutrient supplied by anemonefish may be phosphate, which can become limiting to zooxanthellae when ammonia availability is artificially elevated (Fitt and Cook, 2001). Our preliminary studies indicate that anemonefish excrete phosphate at very slow rates which do not apparently meet the phosphate uptake needs of the zooxanthellae in this association. Starved host anemones that have been cultured with phosphate supplements do not remove additional phosphate from seawater, and appear to be phosphate sufficient, while those that have been cultured with anemonefish rapidly absorb proffered phosphate, and appear to suffer from a deficiency of this nutrient (Godinot and Chadwick, unpublished data). Phosphates are present in reef fish feces (Meyer and Schultz, 1985a), and resident fish could contribute phosphate and other nutrients to host anemones through this pathway. The more-or-less constant supply of nutrients released by anemonefish during the daytime (Roopin et al., 2008) through both pathways may provide a more easily-utilized source of nutrition than the once-daily ammonia spike that we provided. Nutritional benefits to malnourished hosts through fish excretions also may extend throughout the night (Roopin et al., 2008), because starved zooxanthellae also can enhance their dark carbon fixation in the presence of elevated ammonia concentrations (Cook et al., 1992).

Our anecdotal observations on anemone behavior indicated that this aspect of host biology also differed among the experimental treatments. The anemones inhabited by resident anemonefish remained attached to their rocks and rarely changed location in the tanks. In contrast, the control anemones frequently moved about their tanks, climbed on the tank walls, and retracted their tentacles. This tentacle contraction was intermittent and did not affect size measurements, which always were made when anemones were fully expanded. Near the end of the starvation experiment, anemones in the ammonia treatment also exhibited some of these behaviors (M. Roopin, pers. obser.). These behavioral differences suggest that anemones cultured with fish may expand their tentacles more frequently than those without, as observed also in field populations (Porat and Chadwick-Furman, 2004). The causes of enhanced tentacle expansion by hosts with anemonefish are not known, but may relate to physical stimulation of the host by fish swimming movements among the host tentacles.

We conclude that anemonefishes provide major nutritional benefits to host sea anemones, including enhanced host physiological condition and prevention of shrinkage, through fish excretion of constantly-renewed ammonia. Variation in planktonic food supply on coral reefs can compromise host anemone nutritional state, leading to a reduction in zooxanthella populations and in extreme cases even destabilization of the host-algal association (Smith and Muscatine, 1999). By excreting ammonia at a constant basal rate for many hours after feeding (Roopin et al., 2008), resident anemonefishes can fertilize host anemones and buffer fluctuations in environmental nutrient conditions that otherwise negatively impact host growth and survival.

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